EFFECTS OF LOPERAMIDE ON ACETYLCHOLINE AND PROSTAGLANDIN RELEASE FROM ISOLATED GUINEA PIG ILEUM

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Abstract—Loperamide, an effective antidiarrheal agent, was investigated in attempts to determine the site of action which underlies the antiperistaltic and other antidiarrheal actions. In in vitro studies, this compound applied in a dose over \(10^{-8}\) g/ml, inhibited the release of both acetylcholine and prostaglandins during circumferential distension of the intestinal wall, in a dose dependent manner. The inhibited acetylcholine release, but not prostaglandin release, was reversed by naloxone. This suggests that loperamide inhibits acetylcholine release by interacting with opiate receptor sites in the myenteric plexus. The inhibition of prostaglandin release may be due to inhibition of prostaglandin synthesis in the intestine because loperamide prevented the biosynthesis of prostaglandin from arachidonic acid. Although a high concentration of loperamide \(10^{-6}\) g/ml inhibited the contraction of the intestine to acetylcholine, this compound inhibited the contraction to nicotine and serotonin at a concentration which had no effect on the contraction to acetylcholine. Thus loperamide apparently inhibits the peristaltic movement principally by reducing the release of acetylcholine and prostaglandin, at least during circumferential distension of the intestinal wall in vitro. The finding that loperamide inhibited the biosynthesis of prostaglandin may lead to elucidation of the mechanism of its antidiarrheal activity.

Loperamide has been demonstrated to be a specific and safe antidiarrheal agent. It has a wide margin of safety and exhibits antidiarrheal effect without opiate-like central nervous effects (1, 2, 3). Pelemans and Vantrappen (4) reported that the efficacy of the drug could be demonstrated in patients with ‘functional’ diarrhea due to an irritable colon as well as in patients with diarrhea as the result of organic lesions. A diarrhea induced by prostaglandin E\(_1\) was also prevented by loperamide (5). The pharmacology of loperamide differs significantly from that of other agents in that it acts locally to control diarrhea. The mechanism of action has not, however, been fully elucidated.

Many authors have shown that loperamide inhibits peristaltic activity in vitro (6, 7) and in vivo (8, 9). Van Nueten et al. (6) suggested that the drug interacts with both cholinergic and non-cholinergic mechanisms involved in peristaltic activity. Inhibition of the peristaltic reflex by the drug can be reversed partially by prostaglandin E (10) which is considered to modulate the activity of the cholinergic nervous system in the myenteric plexus (11, 12). Recently, release of acetylcholine (ACh) (13, 14) and prostaglandins (15) has been demonstrated during the distension of the intestinal wall and the importance of these
agents in the peristalsis was discussed. Distension of the intestinal wall is also known to stimulate the myenteric plexus of the small intestine (16, 17).

The possibility that loperamide induces a decrease in the ACh release from nerves involved in peristalsis was investigated herein.

MATERIALS AND METHODS

Male guinea pigs weighing 500 to 700 g were used. Four to eight segments of the intestine about 5 cm long were excised from the distal part of the small intestine, excluding the most distal 10 cm portion, and weighed after blotting both sides of the wall with filter paper. These preparations were preserved in Tyrode solution for 20 min at room temperature and then for 15 min in ice cold Tyrode solution with precautions for prevent possible existence of free ACh which could be released during preparation of the gut segments. The segments were then transferred to 5 μM eserine containing Tyrode solution and incubated separately in organic baths containing 7 to 10 ml oxygenated solution at 38°C for 10 min under any one of the procedures described below.

The procedures tested were—

a) Distension in circumferential direction (C): The segment was distended by inserting a glass rod of proper diameter, usually 7 mm, into the intestinal lumen and then incubation was carried out in the presence or absence of loperamide.

b) Undistension (N): The segment was incubated without any treatment such as distension in the presence or absence of loperamide. In the experiments with loperamide, the intestinal segments which had been pretreated with the drug for 10 min during the period of ice cold preservation were used because the maximal effects of the drug to the intestinal movement were obtained a few minutes after application of the drug (6) while the effect of distension on the ACh release was evident within 30 sec (18). At the end of experiments, the bathing solution was collected and tested for ACh and prostaglandin-like activity.

Assay of ACh

The collected bath fluid was gently shaken with one tenth volume of Amberlite XAD-2 (19) for 10 min and centrifuged in order to remove loperamide and prostaglandins. This procedure did not produce any loss of ACh in the bath fluid. Thereafter, ACh was determined by assaying an aliquot of the thus treated bath fluid on a longitudinal muscle strip of guinea pig ileum sensitized with 50 nM eserine. To prevent the release of endogenous ACh from the longitudinal muscle strip, morphine hydrochloride (10 μM) was added to the Tyrode solution of the assay bath. The active substance in the bath fluid was identified as ACh based on the following evidence. 1) The contraction of muscle strip induced by bath fluid was always abolished by atropine (0.1 μg/ml). 2) The active substance lost its activity after boiling the bath fluid for a few minutes in alkaline. The ACh release was expressed in ng/g tissue/min.

Assay of prostaglandins

Prostaglandin in collected bath fluid was assayed on a rat fundus strip (20) suspended in oxygenated Tyrode solution at 37°C. To increase the specificity of the preparation to
prostaglandin, hyoscine, phenoxybenzamine, pyrilamine (0.1 \mu g/ml in each case), inderal
2 \mu g/ml and methysergide 10 ng/ml were added to the Tyrode solution. The contractions
of the fundus strip to the bath fluid were compared with those of known standard doses of
prostaglandin E\(_2\). The amount of released prostaglandins was expressed as ng prostaglandin
E\(_2\) equivalent/g tissue/min, although the bath fluid contained prostaglandin E\(_2\) and F\(_2\alpha\)
as shown below. When the intestinal segment was exposed to drugs such as loperamide
or naloxone, the bath fluids were compared with standard prostaglandin solutions to which
the drugs concerned were added to give the same final concentration in the assay bath.

In some experiments, dried bath fluid was dissolved in ether and subjected to thin layer
chromatography on silica gel G containing 3 per cent silver nitrate, as described by Bennett
et al (21). The \(A\) \(H\) solvent system of Green and Samuelsson (22) was used as the developing
solvent. Authentic prostaglandin E\(_1\), E\(_2\) and F\(_2\alpha\) were treated similarly and chromatogra-
phed concurrently. Centimeter bands of the plates were then extracted with 1 ml of 50 per
cent chloroform in methanol. After separation from solid matter, the solvent was evaporated
at a temperature below 30°C and the residue was shaken with 1 ml of Tyrode solution for
biological assay. As a result, the prostaglandin-like substance in the bath fluid was
identified as prostaglandin E\(_2\) and F\(_2\alpha\).

**Prostaglandin biosynthesis**

Enzyme preparation for prostaglandin biosynthesis was obtained by the method of
Willis et al (23) as follows; freshly isolated guinea pig small intestine (wet weight ap-
proximately 10 g) was rapidly frozen in liquid nitrogen and crushed to a fine powder. The
powdered tissue was suspended in Tyrode solution (30 ml) and stored in ice for up to 20 min
before use. The samples of the powdered ileum suspension (5 ml) were incubated aerobically
with arachidonic acid (added in a 5 \mu l volume of benzene, final concentration 20 \mu g/ml)
for 20 min at 37°C. Loperamide (in 50 \mu l Tyrode solution) at a final concentration of
10\(^{-8}\) to 10\(^{-6}\) g/ml was added 5 min prior to the arachidonic acid; Tyrode solution only was
added to the controls. Twenty min after addition of the arachidonate, 1 ml samples of the
ileum powder suspension were withdrawn and immediately shaken with 4 ml acidic saline.
Prostaglandins were extracted twice from the acidified samples (pH 2.5 to 2.8) with an equal
volume of ethylacetate, and then the extracts were dried at 40°C under reduced pressure.
The material in each extract was dissolved in Tyrode solution and assayed on a rat fundus
strip.

**Chemicals**

Prostaglandin E\(_1\), E\(_2\), F\(_2\alpha\) (Ono), acetylcholine chloride (Sigma), phenoxybenzamine
hydrochloride (Tokyo Kasei), propranolol hydrochloride (Sumitomo), pyrilamine maleate
(K & K), methysergide maleate (Sandoz), naloxone (Sankyo), morphine hydrochloride
(Takeda), physostigmine salicylate (Merck), Amberlite XAD-2 (Rohm & Hass) and loper-
amide (Janssen). All other chemicals were of analytical grade.
RESULTS

Inhibition of ACh release

Treatment of the guinea pig ileum with loperamide inhibited the ACh output during the resting state. The extent of reduction of ACh release depended on the concentration of loperamide; by $10^{-8}$ g/ml loperamide the release of ACh was reduced from $9.0\pm0.9$ to $6.9\pm0.7$ ng/g/min (mean±S.E.), by $10^{-7}$ g/ml to $4.7\pm0.5$ and by $10^{-6}$ g/ml to $4.2\pm0.8$ ng/g/min, respectively. In a previous paper (18) it was shown that the release of ACh from the intestine was significantly increased by circumferential distension of the intestinal wall. The ACh release induced by distension was also reduced in the presence of loperamide in a concentration-dependent manner. This release was reduced by ($10^{-8}$, $10^{-7}$ and $10^{-6}$ g/ml) loperamide from $20.1\pm1.8$ to $13.7\pm1.1$, $10.8\pm0.8$ and $8.1\pm1.2$ ng/g/min, respectively (Fig. 1).

Inhibition of prostaglandin release

When the intestinal segments were distended circumferentially, the release of prostaglan-
andin into the bathing fluid was enhanced from $1.7\pm0.1$ ng/g/min at rest to $7.0\pm0.3$ ng/g/min. When loperamide was added to the medium in the concentration of $10^{-9}$, $10^{-7}$ and $10^{-6}$ g/ml, the prostaglandin release during distension was reduced to $6.2\pm0.3$, $5.2\pm0.4$ and $5.0\pm0.4$ ng/g/min respectively, while the release during the resting state was only slightly reduced (Fig. 2).

**Effect of loperamide in the presence of naloxone**

Recently, Mackerer et al (9) concluded that loperamide binds to opiate receptor sites in the myenteric plexus of the small intestine. If so, it is conceivable that loperamide exhibited the above mentioned effects by interacting with opiate receptors. In order to examine this possibility, the intestinal segments were pretreated with $10^{-8}$ g/ml naloxone for 15 min under ice cold condition and then used for the experiments. The experiments were carried out in the presence of naloxone. In these cases, the inhibitory effects of loperamide on the distension-induced release of ACh were not observed (Fig. 3A). This concentration of naloxone applied alone had no effect on ACh release, but $10^{-7}$ g/ml of naloxone increased the release. Increased liberation of ACh from isolated intestine by naloxone has also been shown by Waterfield and Kosterlitz (24) but the effective concentration of naloxone in their work was somewhat lower ($3.3\times10^{-8}$ g/ml).

The inhibitory effect of the loperamide on the distension-induced release of prostaglandin on the other hand, was not affected by naloxone (Fig. 3B).

**FIG. 3.** Effect of naloxone on the inhibition of ACh- and prostaglandin-release by loperamide during distension. A; ACh release. B; Prostaglandin release. Control; Circumferentially distended segment without drug. Nalx; naloxone $10^{-8}$ g/ml. Lop; loperamide $10^{-8}$ g/ml. Vertical bars represent S.E. *P<0.05, **P<0.01
Inhibition of prostaglandin biosynthesis by loperamide

With no pre-incubation period, loperamide inhibited prostaglandin biosynthesis as illustrated in Fig. 4. The inhibition was 19.0±8.5 and 44.8±4.0% at 10^-7 and 10^-6 g/ml loperamide respectively.

Effect of loperamide on the response of the ileum to spasmogenic drugs

The responses of the longitudinal muscle strips of guinea pig ileum to the three agonists, ACh, nicotine and serotonin, were studied by recording the isotonic contraction of the preparations before and after treatment with loperamide. After control responses had been obtained, loperamide was added to the bathing solution and was contacted with tissue for at least 10 min. Thereafter the contractions to each agonist were repeated in the presence of loperamide. A concentration of 0.1 µg/ml loperamide considerably reduced nicotine-induced contractions of ileum and less intensely those to serotonin. This concentration of loperamide had no effect on the contraction to ACh (Table 1, Fig. 5). Addition of PGE2 (1 ng/ml) in the presence of loperamide partly restored the responses to nicotine and serotonin. Contraction to ACh was slightly enhanced by PGE2.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Loperamide % of control ±S.E.</th>
<th>Loperamide+PGE2 % of control ±S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotine</td>
<td>11± 6 (5)</td>
<td>27± 7 (5)</td>
</tr>
<tr>
<td>5-HT</td>
<td>34±11 (5)</td>
<td>62±10 (5)</td>
</tr>
<tr>
<td>ACh</td>
<td>93± 2 (10)</td>
<td>114± 5 (10)</td>
</tr>
</tbody>
</table>

Submaximal contractions to agonists were recorded in the presence of loperamide 10^-7 g/ml or loperamide (10^-7 g/ml) + PGE2 (10^-3 g/ml). The concentrations of agonists used were; nicotine 10^-6, 5-HT 10^-6 and ACh 10^-8 g/ml. Figures in parentheses indicate number of experiments.
FIG. 5. Effect of loperamide on the mechanical responses to agonists and its reversal by PGE2. Lop; loperamide 10^{-7} g/ml. PGE2; prostaglandin E2 10^{-9} g/ml. w; washed out three times with fresh Tyrode solution.

At a concentration of 1.0 \mu g/ml, loperamide inhibited contractions to all three agonists including ACh. The loperamide-induced inhibitory effects remained even with replacement of the bath fluid with fresh Tyrode solution, suggesting that this compound firmly binds to intestinal tissue.

DISCUSSION

It is well known that the distension of the intestinal wall plays a significant role in evoking peristalsis. The present work showed that loperamide inhibited the distension induced release of ACh and prostaglandins. Together with the observation of Nueten et al (6) that loperamide produced a sustained inhibition of the peristaltic activity of guinea pig ileum, our present results favor the view that the release of ACh and prostaglandins by circumferential distension of the intestinal wall plays an important role in the peristalsis (13, 14, 15).

The inhibitory effect of loperamide on ACh release can be explained by its interaction with opiate receptor site in the myenteric plexus since the effects were completely reversed by the pretreatment of the preparations with naloxone and since it has been shown that the ACh release during distension of the intestinal wall originated from the myenteric plexus (18).

High concentration of loperamide (10^{-8} g/ml) inhibited the contraction of longitudinal muscle strip to ACh, suggesting that loperamide acts directly on the smooth muscle. Thus, the drug seems to act both on nervous elements and on smooth muscle itself, at high concentrations. No doubt, peristalsis can be prevented by inhibiting contraction of smooth muscle. The effect of loperamide on smooth muscle, however, is assumed to be less important than that on the nervous activity, regarding the effect of this compound on peristaltic movement. The concentration of loperamide required to inhibit the contraction to ACh (direct effect) was more than 100 times higher than that required to reduce the distension-
induced release of ACh (effect on the nervous activity). There was no great discrepancy
between the concentration of loperamide required to inhibit the nervous activity and that
required to produce a sustained inhibition of the peristaltic activity in guinea pig ileum (6).

Concerning the mechanism of reversal of loperamide inhibition on serotonin- and
nicotine-induced contractions of guinea pig ileum by PGE₂, two sites of action of PG can
be considered; a neurotropic one in the myenteric plexus and musculotropic one on the
smooth muscle. Actually PGE₂ as low as 5 ng/ml increased the release of ACh from the
isolated small intestine induced by nicotine or serotonin (12). PGE₂ seems, however,
unlikely to be concerned only with recovery of the neuronal release of ACh since responses
to ACh were slightly enhanced in the presence of loperamide by PGE₂ (from 93 to 114% of
control as shown in Table 1), and because PGE₂ itself enhanced the contractions to ACh
(25, 26). Therefore, the effect of PGE₂ may be related both to an increased ACh release
and sensitizing action on smooth muscle.

The antidiarrheal activity of loperamide could be partly explained by its effect on
peristalsis. The observation, however, that atropine which is most effective in reducing
peristalsis is not an effective antidiarrheal drug (27) suggests that inhibition of peristaltic
activity alone is not sufficient to prevent diarrhea. Although diarrhea related mechanisms
have not been fully defined, prostaglandins have been proposed as important agents re-
sponsible for the diarrhea of diverse origin for the following reasons; a) prostaglandin
induces secretion of fluid into the small intestine which is compatible with that found in
acute diarrhea (28, 29); b) indomethacin, an effective inhibitor of prostaglandin biosyn-
thesis, prevented cholera-induced diarrhea (30); and c) increased local synthesis of pro-
staglandins occurs in diarrhea associated with inflammatory bowel disease (31) or with amino-
peptide secreting tumors (32). In considering prostaglandins as a possible mediator of
diarrhea, the inhibition of prostaglandin biosynthesis may be involved in the antidiarrheal
action of loperamide. The possibility is supported by the effectiveness of the drug in patients
with diarrhea due to irritable colon (3).

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